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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Suppresses Tumor Necrosis Factor-α and Anti-CD40–Induced Activation of NF-κB/Rel in Dendritic Cells: p50 Homodimer Activation Is Not Affected

CARL E. RUBY, MARK LEID,[1] and NANCY I. KERKVLIET

Department of Environmental and Molecular Toxicology, and Environmental Health Science Center, Oregon State University, Corvallis, Oregon

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) suppresses many immune responses, both innate and adaptive. Suppression is mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor. The AhR mediates TCDD toxicity presumably through the alteration of transcriptional events, either by promoting gene expression or potentially by physically interacting with other transcription factors. Another transcription factor, NF-κB/Rel, is involved in several signaling pathways in immune cells and is crucial for generating effective immune responses. Dendritic cells (DCs), considered to be the "pacemakers" of the immune system, were recently recognized as targets of TCDD and are also dependent on NF-κB/Rel for activation and survival. In these studies, we investigated whether TCDD would alter the activation of NF-κB/Rel in DCs. The dendritic cell line DC2.4 was exposed to TCDD before treatment with tumor necrosis factor α (TNF-α) or anti-CD40, and NF-κB/Rel activation was measured by electrophoretic mobility shift assay and immunoblotting. TCDD suppressed the binding of NF-κB/Rel to its cognate response element in TNF-α– and anti-CD40–treated cells and blocked translocation to the nucleus. The AhR was shown to associate with RelA, after coimmunoprecipitation, and seemed to block its binding to DNA. It is noteworthy that p50 homodimers freely bound to DNA. These results suggest that TCDD may alter the balance between NF-κB/Rel heterodimers and transcriptional inhibitory p50 homodimers in DCs, leading to defects in the DCs and suppression of the immune response.

The immune system is a sensitive target of TCDD, a widespread environmental contaminant that induces many biochemical and pathological effects (Kerkvliet and Burleson, 1994). Numerous studies linking TCDD exposure to an increase in susceptibility to various pathogens and to the suppression of humoral- and cell-mediated immune responses in mice verify the impact of TCDD on the immune system (Kerkvliet and Brauner, 1987; Kerkvliet and Baecher-Steppan, 1988; House et al., 1990). TCDD toxicity is primarily mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated basic-helix-loop-helix transcription factor (Rowlands and Gustafsson, 1997). Binding of TCDD to AhR in the cytoplasm initiates shedding of two 90-kDa heat-shock proteins and an immunophilin protein complexed with AhR, allowing for the rapid translocation of AhR into the nucleus. The activated AhR, after dimerization with aryl hydrocarbon receptor nuclear translocator, promotes the expression of various genes through an interaction with specific regions of DNA called dioxin-response elements. In contrast to the direct influence on gene expression via the dioxin-response elements, the AhR might also alter gene expression through indirect means as seen in the increased expression of the transcription factor AP-1 (Puga et al., 1992). Additional effects of the AhR on transcription factors include cross-talk with the estrogen receptor (Klinge et al., 2000) and physical association with retinoblastoma protein (Ge and Elferink, 1998) and RelA (Tian et al., 1999).

The transcription factor NF-κB/Rel is intimately involved in the immune system (Baldwin, 1996; Ghosh et al., 1998). NF-κB/Rel consists of a family of five proteins—p50, p52, RelA, RelB, and c-rel—forming various DNA-binding homo- and heterodimeric complexes. The Rel proteins—RelA, RelB, and c-rel—share a conserved NH_2 terminus identified as the

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[1] Current address: Laboratory of Molecular Pharmacology, Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University.

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; DC, dendritic cell; TNF-α, tumor necrosis factor α; PAGE, polyacrylamide gel electrophoresis; TRAF, tumor necrosis factor receptor-associated factor; EMSA, electromobility shift assay; Dex, dexamethasone; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; PAB, phosphate-buffered saline, 1% fetal bovine serum, 0.1% sodium azide.

Rel homology domain and a nonconserved COOH terminus containing a transcriptional activation domain. In contrast, p50 and p52 lack transcriptional activation domains, forming homodimer complexes that may inhibit transcription (Lernbecher et al., 1993). The activation of NF-κB/Rel is regulated by a battery of inhibitor proteins called IκBs that block the nuclear localization signal, leading to cytoplasmic sequestration. Phosphorylation of the inhibitor protein on specific serine moieties directs its degradation, allowing NF-κB/Rel to translocate to the nucleus and influence gene expression. The immune system is dependent on NF-κB/Rel for the transcription of some of its most critical genes, including cytokines and signaling proteins. Moreover, the dependence of the immune system on NF-κB/Rel is apparent in mice with deleted NF-κB/Rel. Such knockout mice display a wide range of immune defects, from suppressed humoral- and cell-mediated immunity to a profound loss of dendritic cells (DCs) (Sha et al., 1995; Doi et al., 1997; Wu et al., 1998).

TCDD-exposed mice show many similarities to NF-κB/Rel knockout mice. However, apart from the essential role of the AhR, the biochemical and cellular mechanisms underlying TCDD immunotoxicity have yet to be elucidated. A potential mechanism of toxicity could be via alterations in NF-κB/Rel. DCs have recently been shown to be a target of TCDD toxicity (Shepherd et al., 2001; Vorderstrasse and Kerkvliet, 2001), providing a highly relevant model for the study of this potential mechanism of TCDD immunotoxicity. DCs are integral in the regulation of the immune system as the most potent antigen-presenting cells, inducing and maintaining immune responses (Banchereau and Steinman, 1998). Furthermore, on a molecular level, DCs rely on NF-κB/Rel to mediate their differentiation, maturation, and survival (Oyama et al., 1998; Rescigno et al., 1998; Verhasselt et al., 1999).

In the studies presented here, we investigated the effects of TCDD on the activation of NF-κB/Rel in DC by TNF-α and anti-CD40, both of which are known to activate NF-κB/Rel (Baldwin, 1996; Ghosh et al., 1998). We used a DC line, DC2.4, and used DNA binding assays and various other immunoblot techniques to determine the influence of TCDD on the activation of NF-κB/Rel. Our results show that TCDD suppresses DNA binding and nuclear translocation of NF-κB/Rel in TNF-α– and anti-CD40–activated DC2.4 cells. This suppression seemed to be mediated predominantly by an association between the AhR and RelA, inhibiting the binding of NF-κB/Rel heterodimers to DNA. In contrast, p50 homodimer binding was unaffected by TCDD. These results suggest that TCDD may alter the balance between NF-κB/Rel heterodimers and transcriptional inhibitory p50 homodimers in DCs, leading to defects in DCs and suppression of the immune response.

Materials and Methods

Reagents and Antibodies. Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Recombinant mTNF-α was purchased from Peprotech (London, United Kingdom). TCDD (≥99% pure) was purchased from Cambridge Isotope Laboratories (Woburn, MA). All other reagents and cell culture supplies were purchased from Invitrogen (Carlsbad, CA). Dr. Tony Vella (Oregon State University, Corvallis, OR) provided anti-CD40 (FGK45.5) antibodies. Anti-murine AhR antibodies (3–14B) were provided by Dr. Alan Poland (National Institute for Occupational Safety and Health, Morgantown, WV) and were raised against synthetic peptides corresponding to the N terminus of the C57Bl/6 mouse liver AhR (A. Poland, personal communication). Antibodies against RelA, RelB, c-rel, p50, p52, IκBα, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-IκBα was purchased from Cell Signaling Technology (Beverly, MA). The pcDNA3/βmAhR-FLAG construct was a gift from Dr. Gary Perdew (Pennsylvania State University, University Park, PA). Dexamethasone (Dex) was purchased from Sigma (St. Louis, MO).

Cell Culture. The cell line DC2.4, derived from C57Bl/6 mice, was provided by Dr. Kenneth L. Rock (Division of Lymphocyte Biology, Dana Farber Cancer Institute, Boston, MA) (Shen et al., 1997). DC2.4 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 10 mM Na-pyruvate, and 50 mg/ml gentamicin. Cultures were grown to 75% confluence, treated with TCDD (10^{-9} M) for 24 h, and activated with TNF-α (10 ng/ml) or anti-CD40 (25 μg/ml) for 2 h before harvesting. Cells were treated with Dex (10^{-9} M) as a positive control for 2 h before the addition of TNF-α and harvested after 2 h.

Whole Cell Lysates and Subcellular Fractionation. Whole cell lysates were prepared by incubating DC2.4 cells in 10 nM Tris, pH 7.4, 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT, 3 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 20 min at 4°C. Samples were centrifuged at 15,000 rpm in a microcentrifuge, 4× SDS-PAGE sample buffer was added to the supernatant, and extracts were placed in boiling water for 5 min.

Nuclear and cytoplasmic extracts were prepared as described previously (Dyer and Herzog, 1995). Briefly, cell pellets were resuspended in sucrose buffer (0.32 M sucrose, 3 mM $CaCl_2$, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM magnesium acetate, 1 mM DTT, and 0.5 mM PMSF) with 0.5% (v/v) IGEPAL nonionic detergent (Sigma) by gentle mixing with a pipette and then were centrifuged. To the cytoplasmic fraction, 0.22 volumes of 5× cytoplasmic extraction buffer (0.15 M HEPES, 0.7 M KCl, and 0.015 M $MgCl_2$) was added. The cytoplasmic fraction was then centrifuged at 15,000 rpm, and the supernatant was transferred to a fresh tube containing 25% v/v glycerol and stored at −80°C. The nuclei were washed twice in sucrose buffer without IGEPAL. Nuclei were resuspended in low-salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF), and then 1 volume of high-salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM KCl, 0.2 mM EDTA 1% IGEPAL, 0.5 mM DTT, and 0.5 mM PMSF) was carefully added in one-quarter increments. Nuclei were incubated on ice for 30 min, diluted to a ratio of 1:2.5 with diluent (25 mM HEPES, 25% glycerol, 0.1 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF), and centrifuged at 15,000 rpm in a microcentrifuge at 4°C. Nuclear lysates were stored at −80°C.

DNA Binding Assay. Electrophoretic mobility shift assay (EMSA) was used to assess sequence-specific binding of DC2.4 nuclear NF-κB/Rel to DNA (Dyer and Herzog, 1995). Briefly, a synthetic 20-basepair consensus κB-RE probe (upper strand, 5′-GATCGGCAGGGGAATTCCCC-3′; lower strand, 5′-GATCGGGGAATTCCCCTGCC-3′) was labeled with α-[^{32}P]dATP using Klenow fragment (Invitrogen) and then was used for DNA binding assays. Nuclear extracts were prepared as described above. Samples (5 μg) were incubated with binding buffer (12 mM HEPES, pH 7.3, 4 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 20 mM DTT, and 1 mg/ml bovine serum albumin), 4 μg of poly-dI-dC (Amersham Biosciences, Piscataway, NJ) and 100,000 cpm of ^{32}P-labeled κB-RE for 20 min at room temperature. For supershift analysis, antibodies to RelA, RelB, c-rel, p50, and p52 were added to the reaction mixture according to the manufacturer's protocol (Santa Cruz Biotechnology) and incubated for 10 min at room temperature. Samples were analyzed on a 5% polyacrylamide gel in 0.5% Tris/borate/EDTA (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) and visualized by autoradiography.

Immunoblotting. Cell extracts were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol using a Genie Electroblotter (Idea Scientific Inc., Minneapolis, MN). Membranes were blocked overnight at 4°C in TBS (25 mM Tris, pH 7.4, and 150 mM NaCl) containing 5% nonfat dry milk. Antibodies were diluted

in TBS containing 1% nonfat dry milk, and the membranes were incubated with primary antibodies for at least 1.5 h at room temperature. The primary antibodies, anti-RelA, anti-RelB, anti-c-rel, anti-actin, anti-IκBα, and anti-phospho-IκBα, were used according to the manufacturer's instructions. Horseradish peroxidase–conjugated secondary antibodies, donkey anti-rabbit IgG and goat anti-mouse IgG, were used according to the manufacturer's instructions. After each antibody treatment, blots were washed three times in TBS containing 0.05% Tween 20. Antibody complexes were visualized with the use of chemiluminescence (Pierce Chemical, Rockford, IL).

Transient Transfections. DC2.4 cells were transfected at 80% confluence in 25-cm^2 tissue culture flasks by a LipofectAMINE procedure as specified by the manufacturer (Invitrogen). The cells were transfected with pcDNA3/βmAhR-FLAG (a gift from Dr. Gary Perdew, Pennsylvania State University, University Park, PA). Transfection efficiency was determined to be approximately 35% by intracellular staining and analysis by fluorescence-activated cell sorting (data not shown). The transfected cells were harvested with trypsin-EDTA and washed once in phosphate-buffered saline.

Intracellular Staining and Flow Cytometry. DC2.4 cells were collected and washed in cold PAB (phosphate-buffered saline, 1% fetal bovine serum, 0.1% sodium azide) and then washed in PAB-0.05% saponin. Cells were treated with mouse IgG or goat IgG to block nonspecific binding, and then appropriate purified RelA, RelB, c-rel, p50, or p52 antibodies were added, followed by the addition of fluorochrome-conjugated streptavidin and secondary antibodies. Fluorescein isothiocyanate–labeled anti-actin antibody was used as a positive control (Sigma, St. Louis, MO). For each sample, at least 10,000 events were collected as listmode data. Listmode data were collected on a Coulter XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA) and analyzed using WinList software (Verity Software House, Inc., Topsham, ME).

Coimmunoprecipitation. Cytosolic lysate isolated from pcDNA3/βmAhR-FLAG–transfected cells was immunoprecipitated with streptavidin magnetic beads (Dynal Biotech, Oslo, Norway) coated with biotinylated anti-FLAG antibody (Sigma, St. Louis, MO). As a control, magnetic beads without anti-FLAG antibody were used. Cytosol in buffer 1 (0.32 M sucrose, 3 mM $CaCl_2$, 0.1 mM EDTA, 10 nM Tris-HCl, pH8.0, 1 mM DTT, 0.5 mM PMSF, 2 mM MgAc, and 0.5% IGEPAL) was rotated for 2 h with 25 ml of anti-FLAG magnetic beads at 4°C. The beads were washed in fresh buffer four times. The beads were then resuspended in 2× SDS sample buffer and incubated in boiling water for 5 min. The samples were analyzed for RelA, RelB, and c-rel by immunoblotting.

Results

TCDD Decreases the Binding of NF-κB/Rel in TNF-α– and anti-CD40–Treated DC2.4 Cells. The binding of TNF-α to CD120 and ligation of CD40 by CD154 are two events that lead to the activation of NF-κB/Rel in DCs (Baldwin, 1996; Ghosh et al., 1998). The CD120 and CD40 receptors induce the activation of NF-κB/Rel via proteins associated with the NH_2 terminus of the receptors called TRAFs. These TRAFs initiate the phosphorylation of IκBs by activating IκB kinases (Rothe et al., 1995), leading to translocation of NF-κB/Rel into the nucleus and binding to κB response elements in DNA. To determine whether TCDD altered the activation of NF-κB/Rel, DC2.4 cells were exposed to TCDD or vehicle control for 24 h and then activated for 2 h with TNF-α or anti-CD40. NF-κB/Rel activation was measured by EMSA.

We identified at least two bands corresponding to NF-κB/Rel binding in DC2.4 cells treated with TNF-α and anti-CD40 (Fig. 1, A and B). TNF-α or anti-CD40 treatment both increased the intensity of the NF-κB/Rel bands (Fig. 1, A and B, lanes 1 versus 3), verifying the capacity of TNF-α and anti-CD40 to activate NF-κB/Rel in these cells. TNF-α induced greater activation of NF-κB/Rel compared with anti-CD40 in several independent experiments. DC2.4 cells exposed to TCDD and then treated with TNF-α demonstrated a decrease in the intensity of the bands corresponding to NF-κB/

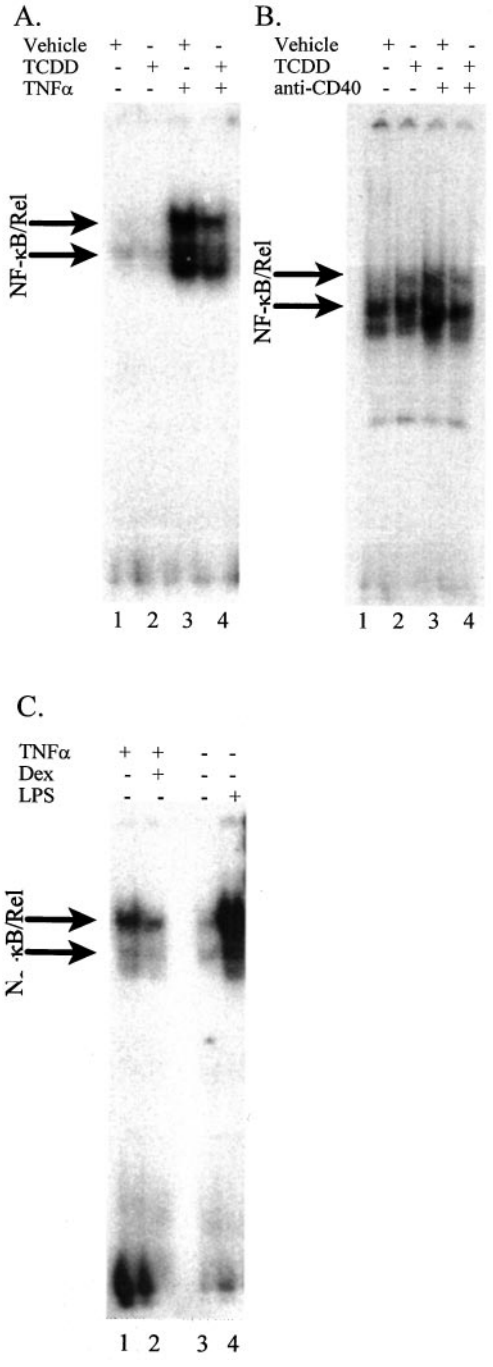

Fig. 1. TCDD suppresses TNF-α– and anti-CD40–induced NF-κB/Rel activation. A, nuclear extracts from cells exposed to 10^{-9} M TCDD or dimethyl sulfoxide (0.01%) for 24 h and then treated with 10 ng/mL TNF-α for 2 h were used for EMSA. A κB-RE sequence was used to detect the κB binding activities. Samples were separated by electrophoreses (1.5 h). B, nuclear extracts from cells exposed to 10^{-9} M TCDD or dimethyl sulfoxide (0.01%) for 24 h and then treated with 25 μg/mL anti-CD40 (FGK45.5) for 2 h were used for EMSA. A κB-RE sequence was used to detect the κB binding activities. Samples were separated by electrophoresis (2.0 h). C, nuclear extracts from cells treated with 10^{-6} M Dex 1 h before treatment with 1 ng/mL TNF-α were used as a control (lanes 1 and 2). The results are representative of two or more separate experiments.

Rel binding DNA when compared with vehicle-treated controls (Fig. 1A, lanes 3 versus 4). We also observed a decrease in NF-κB/Rel binding in DC2.4 cells exposed to TCDD and activated for 2 h with anti-CD40 (Fig. 1B, lanes 3 and 4). In Fig. 1B (lanes 1 and 2), treatment with TCDD alone increased the intensity of the upper band corresponding to NF-κB/Rel binding to DNA. However, this phenomenon proved to be inconsistent, as seen in Fig. 1A (lane 1 versus lane 2) as well as in additional experiments. As a positive control to demonstrate suppression of NF-κB/Rel activation (Auphan et al., 1995), we treated cells with Dex, which was shown previously to suppress TNF-α–induced NF-κB/Rel activation (Fig. 1C, lanes 1 and 2). In Fig. 1C, lane 4 shows the activation of NF-κB/Rel by LPS, a potent activator (Baldwin, 1996). These results show that TCDD inhibits NF-κB/Rel DNA binding in DC2.4 cells.

Antibody-Supershift Analysis of NF-κB/Rel Binding in TNF-α– and anti-CD40–Activated DC2.4 Cells. The composition of the NF-κB/Rel dimers that corresponded to the two bands seen in the previous EMSA (Fig. 1, A and B) was characterized by antibody supershift. The upper band from TNF-α–activated cells was supershifted by antibodies to RelA, RelB, and p50, but not to c-rel (Fig. 2A). In anti-CD40–activated cells, antibodies to RelA, RelB, and to a lesser extent c-rel supershifted the top band, but p50 antibodies did not (Fig. 2B). In contrast, the lower band was supershifted only by antibodies to p50 (Fig. 2A, lane 5, and Fig. 2B, lane 5). These data indicate that the lower band likely corresponds to p50 homodimer binding, whereas the upper band in TNF-α–treated cells contains Rel/p50 heterodimers.

TCDD Decreased Levels of NF-κB/Rel in the Nucleus after TNF-α Activation. An important step in NF-κB/Rel activation is translocation of the transcription factor to the nucleus. After the proteolysis of IκBα, a member of the IκB inhibitor protein family, NF-κB/Rel, translocates to the nucleus in which it induces gene expression. As a possible explanation for the decreased binding of NF-κB/Rel in TCDD-exposed cells, we analyzed the ability of NF-κB/Rel to translocate to the nucleus after TNF-α activation. Nuclear NF-κB/Rel protein was visualized by immunoblot analysis from DC2.4 cells exposed to TCDD for 24 h and activated for 2 h with TNF-α. In Fig. 3, the levels of RelA, RelB, and to a lesser extent c-rel protein were decreased in the nucleus of TCDD-exposed cells activated with TNF-α compared with vehicle controls. Thus, the effect of TCDD on NF-κB/Rel seems to occur upstream of translocation.

TCDD Does Not Alter Levels of Cellular NF-κB/Rel Protein. Because TCDD treatment decreased NF-κB/Rel binding to DNA and reduced levels of Rel proteins in the nucleus, a possible mechanism for these effects could be a reduction in NF-κB/Rel protein expression. To determine whether TCDD alters the expression of NF-κB/Rel, cellular levels of NF-κB/Rel in DC2.4 cells exposed to TCDD or a vehicle control for 24 h were visualized by immunoblot. Protein levels of NF-κB/Rel in cells exposed to TCDD for 24 h were unaltered when compared with vehicle controls (Fig. 4). In addition, there was no effect of TCDD on NF-κB/Rel in intracellular staining using flow cytometric analysis (data not shown). Thus cellular expression of Rel proteins does not seem to be affected by TCDD.

TCDD Does Not Alter Phosphorylation or Proteolysis of IκBα. NF-κB/Rel is sequestered in the cytoplasm by IκBα, which, when phosphorylated at specific serine residues, earmarks it for destruction (Karin and Ben Neriah, 2000). IκBα degradation permits the translocation of NF-κB/Rel to the nucleus and subsequent binding to DNA. Thus the level of expression of IκBα is a pivotal element in the activation of NF-κB/Rel. This has been demonstrated after Dex treatment, which increases expression of IκBα, leading to the suppression of NF-κB/Rel activation (Auphan et al., 1995). It is possible that TCDD alters either the expression or the phosphorylation of IκB, leading to decreased NF-κB/Rel translocation. We measured the levels of IκBα and levels of the phosphorylated form of IκBα to determine whether TCDD altered these upstream events. Cellular lysates from DC2.4 cells, exposed to TCDD or vehicle for 24 h and then

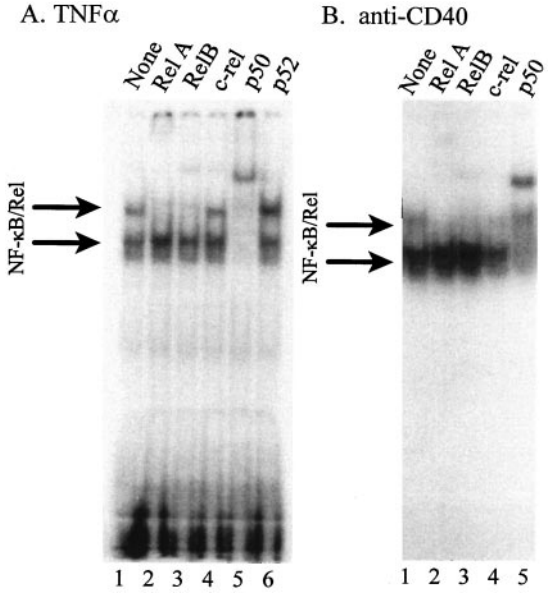

Fig. 2. Antibodies to NF-κB/Rel demonstrate specific binding in supershift analysis of nuclear lysates from DC2.4 cells. Nuclear extracts from cells treated with 10 ng/ml TNF-α (A) and 25 μg/ml anti-CD40 (B) were incubated with specific antisera against the different members of the NF-κB/Rel family before EMSA.

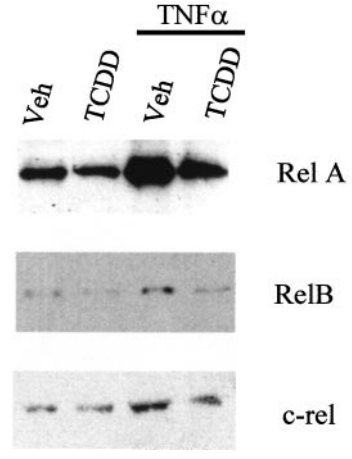

Fig. 3. TCDD suppresses TNF-α–induced nuclear translocation of NF-κB/Rel. Nuclear extracts from DC2.4 cells treated with 10^{-9} M TCDD or vehicle controls 24 h before treatment with 10 ng/mL TNF-α for 2 h were separated on SDS-PAGE and immunoblotted with antibodies specific to RelA, RelB, and c-rel. Gels were loaded with 25 mg of nuclear extract. Results are representative of three separate experiments.

stimulated with TNF-α at various times, were analyzed by immunoblotting.

We were unable to detect the phosphorylated form of IκBα at times earlier than 30 min after TNF-α treatment. In Fig. 5A, cells exposed to TCDD and activated with TNF-α for 30 or 60 min did not display any differences in the phosphorylation of IκBα when compared with vehicle controls. In addition, TCDD altered neither total protein levels of IκBα (Fig. 5B, lane 1 versus lane 2) nor the proteolysis of IκBα at 15, 30, and 60 min after TNF-α treatment (Fig. 5B). It should be noted that the level of IκBα at the 60-min time point seemed to be greater than basal conditions (0 min), possibly because of the ability of NF-κB/Rel to induce its own repressor, IκBα, in a feedback mechanism (Baldwin, 1996). TCDD apparently does not alter IκBα expression, phosphorylation, or destruction.

RelA Interacts with AhR in Transfected DC2.4 Cells. A potential mechanism to explain the suppression of NF-κB/Rel by TCDD is through an association between the AhR and the NF-κB/Rel protein RelA, originally described in hepa1c1c7 cells (Tian et al., 1999). To determine whether the AhR interacted with the proteins of NF-κB/Rel in DC2.4 cells, we immunoprecipitated the AhR and probed for NF-κB/Rel proteins. Cells of the immune system have been shown to express significantly less AhR than the hepa1c1c7 cells used in the aforementioned study (Lawrence et al., 1996; C. E. Ruby, unpublished results). To overcome this deficiency, DC2.4 cells were transfected with an expression vector containing the murine AhR protein fused to a FLAG epitope (Meyer et al., 1998). Cells were transiently transfected for 24 h and analyzed by immunoblot to verify protein expression (data not shown).

Transfected cells were incubated with or without TNF-α for 2 h, and lysates from the cells were immunoprecipitated with magnetic beads coated with anti-FLAG antibodies. Control beads were used to determine nonspecific binding. As shown in Fig. 6, RelA coimmunoprecipitated with transfected AhR. In addition, the amount of RelA that coimmunoprecipitated with transfected AhR in TNF-α–treated cells was increased compared with the levels in untreated controls. This interaction was limited to RelA, because neither c-rel nor RelB seemed to coimmunoprecipitate with transfected AhR. Thus, RelA seems to be the dominant NF-κB/Rel protein to interact with the AhR.

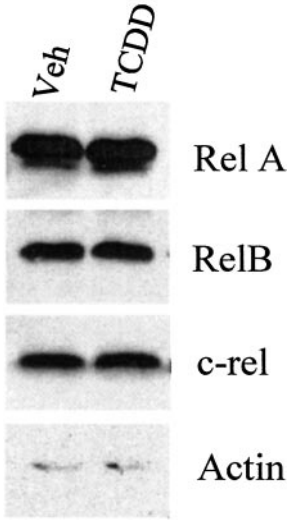

Fig. 4. Protein levels of NF-κB/Rel are not altered by TCDD. Whole-cell extracts from DC2.4 cells treated with 10^{-9} M TCDD or vehicle control were separated by SDS-PAGE and immunoblotted with antibodies specific to RelA, RelB, or c-rel. Results are representative of two or more separate experiments.

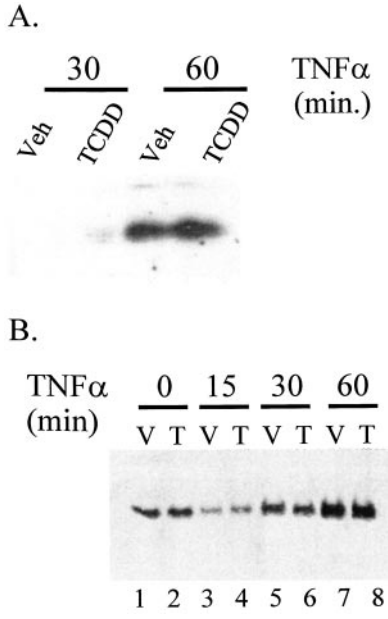

Fig. 5. Proteolysis of IκBα is not altered by TCDD. Cellular extracts from DC2.4 cells treated with 10^{-9} M TCDD (T) or vehicle (V) 24 h before treatment with 10 ng/mL TNF-α for 30 and 60 min (A) or 0, 15, 30, and 60 min (B) were separated by SDS-PAGE and immunoblotted with antibodies specific for the phosphorylated form of IκBα (A) or total IκBα (B). Results are representative of three separate experiments.

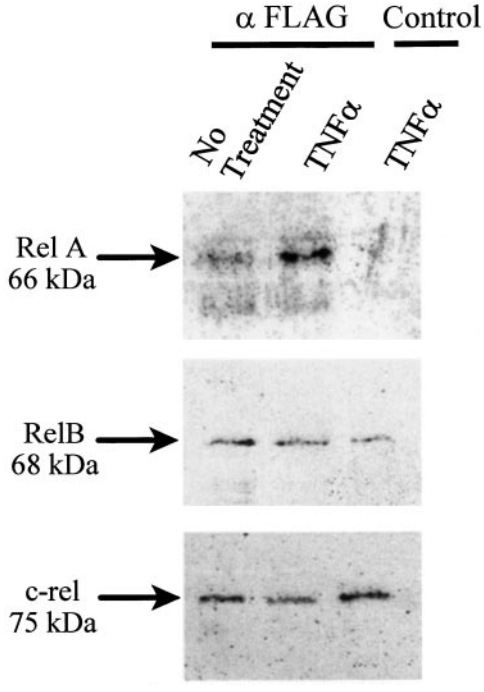

Fig. 6. RelA coimmunoprecipitates with the Ah receptor. DC2.4 cells transiently transfected with FLAG-AhR for 24 h were treated with or without 10 ng/mL TNF-α for 2 h. Cells were lysed, and extracts were incubated with magnetic beads coated with antibodies specific to FLAG or uncoated control beads. Precipitates were separated by SDS-PAGE and immunoblotted with antibodies specific to RelA, RelB, and c-rel. Results are representative of three separate experiments.

Transfected AhR Preferentially Decreased the Binding of NF-κB/Rel. To verify that the AhR, possibly through an interaction with Rel proteins, suppressed NF-κB/Rel binding to DNA, we overexpressed the AhR in DC2.4 cells and measured NF-κB/Rel activation by EMSA. DC2.4 cells were transfected with FLAG-AhR and, after a 2-h incubation with TNF-α, nuclear lysates were generated and analyzed by EMSA. The overexpression of AhR led to a striking loss of the top band (Fig. 7, lanes 3, 4, and 5), previously identified by supershift analysis to consist of RelA/p50 or RelB/p50 heterodimer binding to DNA (Fig. 2). In contrast, the intensity of the lower band seemed to be largely unaffected by overexpression of the AhR. The lower band was supershifted with the addition of antibodies to p50 but not with antibodies specific for RelA, RelB, or c-rel (Fig. 7). These data demonstrate that overexpression of AhR selectively inhibits the binding of Rel/p50 heterodimers, but it does not alter the binding of p50 homodimers.

Discussion

The results from this study demonstrate a suppression of NF-κB/Rel activation by TCDD in a DC line. We showed that TCDD exposure decreased NF-κB/Rel translocation to the nucleus and binding to DNA in DC2.4 cells activated with either TNF-α or anti-CD40, and that this decrease may be mediated by a physical association between the AhR and RelA proteins. These data agree with previously published results establishing the ability of TCDD to suppress the TNF-α–induced activation of NF-κB/Rel in the hepatoma cell line, hepa1c1c7, by means of a physical interaction between AhR and RelA (Tian et al., 1999). Our results also show that p50 homodimer activation is not altered by the AhR. This finding is in partial agreement with a study by Puga et al. (2000) who reported a selective increase in p50 homodimer binding to DNA in the hepa1c1c7 cells after exposure to 5 nM TCDD.

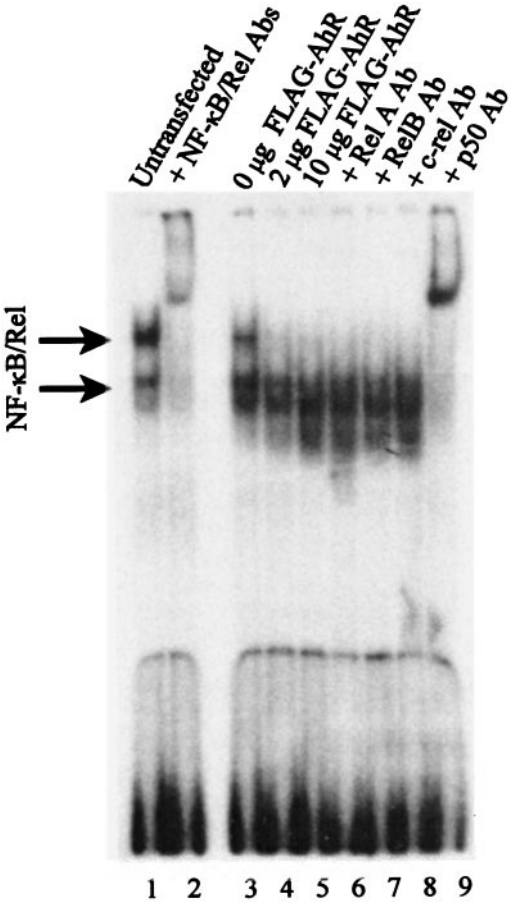

Fig. 7. Overexpression of AhR blocks Rel/p50 heterodimer binding. Nuclear lysates from DC2.4 cells untransfected (lane 1) or transfected with 0, 2, or 10 mg of FLAG-AhR DNA for 24 h and with TNF-α for 2 h (lanes 3–5) were analyzed by EMSA. Supershift analysis of untransfected cells with antibodies specific for NF-κB/Rel (lane 2) and cells transfected with 10 mM FLAG-AhR for 24 h and with 10 ng/mL TNF-α for 2 h using specific antibodies to RelA, RelB, c-rel, and p50 (lanes 6–9). Results are representative of three separate experiments.

In contrast, our results seem to differ with those of Sulentic et al. (2000) and Gollapudi et al. (1998) who reported that TCDD activates NF-κB/Rel in B cells and HIV-infected promonocytes, respectively. Apart from obvious differences in TCDD dose and cell type, a major difference was that these studies analyzed the level of NF-κB/Rel in relatively inactive or resting cells. This is an important point because NF-κB/Rel activity is markedly increased in activated cells, and activated NF-κB/Rel plays a critical role in the induction of an immune response (Sha, 1998). Our studies included the use of TNF-α and anti-CD40, both of which are potent activators of DC and NF-κB/Rel. It was only after these activation stimuli that we observed suppression NF-κB/Rel binding to DNA by TCDD in DC2.4 cells, and these stimuli have been shown to be critical in the function and survival of DC (Rescigno et al., 1998; Miga et al., 2001).

In another study, Kim et al. (2000) demonstrated a physical interaction between the AhR and RelA in human breast cancer cells consistent with our and previous studies. However, they observed enhanced rather than suppressed binding of the AhR-RelA complex to a κB-RE in the c-myc promoter. Because the κB-RE used in the study by Kim et al. (2000) differed significantly from the multimerized consensus κB-RE used in our studies, these findings are difficult to reconcile at this time.

The suppression of NF-κB/Rel and the shift in Rel/p50 heterodimer and p50 homodimer balance shown in this study could be a potential mechanism of TCDD-induced immunotoxicity. Effective immune responses are dependent on DCs, and DC differentiation, maturation, and survival are dependent on NF-κB/Rel activity (Oyama et al., 1998; Rescigno et al., 1998; Verhasselt et al., 1999). Furthermore, alterations in DC function have been shown to lead to immune suppression (Woods et al., 2000). Work done recently in our laboratory has shown that TCDD exposure significantly reduces the number of splenic DCs in mice (Shepherd et al., 2001; Vorderstrasse and Kerkvliet, 2001), and the suppression of NF-κB/Rel conceivably explains this phenomenon. DC development from stem cells in the bone marrow and their maturation rely on the activity of NF-κB/Rel, as demonstrated in NF-κB/Rel knockout mice (Wu et al., 1998). The capacity of TCDD and the AhR to suppress the activation of NF-κB/Rel in DCs could alter their development and/or maturation, thereby reducing the number of DCs in the spleen of TCDD-exposed mice. Survival of the DCs, another event critical in the generation of immune responses, is dependent on the ligation of CD40 and tumor necrosis factor–related activation-induced cytokine (TRANCE), both of which signal through NF-κB/Rel (Josien et al., 2000). Blocking the function of one or both of these molecules leads to unproductive DC–T cell interactions and premature termination of the immune re-

sponse (Miga et al., 2001). Thus immune suppression could be induced through a sequence of events beginning with the decrease in NF-κB/Rel binding in DC by TCDD and culminating in defective DC development, maturation, or survival.

Depending on cell type, the p50 homodimer of NF-κB/Rel has been shown to inhibit rather than promote transcription (Lernbecher et al., 1993). The inhibitory property of p50 homodimers may be related to their ability to bind to DNA, but they fail to introduce a substantial "flexture" or bending of DNA that is important in promoting transcription (Kuprash et al., 1995). DNA-bound p50 homodimers are also unable to recruit a coactivator complex containing CBP or p/CAF, impairing their capability to promote transcription (Sheppard et al., 1999). Our data show that although the AhR can block Rel/p50 heterodimer activity, it seems to have no effect on p50 homodimer binding, thereby potentially shifting the balance between these protein complexes bound to DNA. This phenomenon could also lead to the suppression of gene expression.

In summary, we found that TCDD suppressed TNFα- and anti-CD40-induced activation of NF-κB/Rel in the dendritic cell line, DC2.4. This suppression may result from an association between the AhR and the NF-κB/Rel protein RelA. Overexpression of the AhR did not influence p50 homodimer binding to DNA, suggesting that inhibitory p50 homodimers do not associate with the AhR. This phenomenon may allow for unobstructed binding of p50 homodimers to DNA and possible induction of secondary suppressive effects on transcription. Thus TCDD may affect the function and/or survival of the DC, an important professional antigen-presenting cell, that could lead to extensive immune defects.

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Address correspondence to: Dr. Nancy I. Kerkvliet, 1007 ALS, Oregon State University, Corvallis, OR 97331. E-mail: nancy.kerkvliet@orst.edu